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Different Hematological Phenotypes Caused by the Interaction of Triplicated α-Globin Genes and Heterozygous β-Thalassemia

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The pathophysiology and clinical severity of β-thalassemia are related to the degree of α/non-α-chain imbalance. A triplicated α-globin gene locus can exacerbate effects of excess α-chains caused by a defective β-globin gene, although this is not observed in all cases. Extensive studies on this condition are lacking. We report a group of 17 patients who are heterozygous for both the $\alpha\alpha\alpha^{anti\text{-}3.7}$ allele and a mutation in the β-globin gene cluster. Their clinical phenotypes varied: six had mild anemia with microcytosis and hypochromia, while 11 had more severe anemia with splenomegaly requiring splenectomy (three cases) and blood transfusions (four cases). Different phenotypes were also evident in the presence of the same β-thalassemia mutation: in one family, two individuals had the same α- and β-globin genotypes but presented with different hematologic phenotypes. In addition, the complex interaction involving a triplicated α-globin gene, β^{39}- and δ^{+27}-thalassemia mutations is studied in a family with two siblings presenting with hemolytic anemia, normal Hb A_2 and increased Hb F. Analysis of this series of patients suggests that additional genetic determinants play a role in modulating phenotypic expression in individuals with identical α- and β-globin genotypes. Interaction with a triplicated α-gene can play a role in the clinical presentation of patients with defective β-globin gene expression and should be considered in the diagnosis of atypical cases. Am. J. Hematol. 55:83–88, 1997. © 1997 Wiley-Liss, Inc.

Key words: α-globin gene; β-thalassemia; modifier genes

INTRODUCTION

There is a delicate balance between production of α-and β-globin chains in normal erythropoiesis. Ineffective erythropoiesis, shortening of erythrocyte life span and clinical expression of β-thalassemia are all related to the degree of chain imbalance. Defective β-globin gene expression or additional α-globin genes results in the production of excess α-globin chains, which may precipitate and ultimately lead to the destruction of red blood cells (RBCs) or their precursors [1]. In areas where β-thalassemia mutations are prevalent and heterogeneous, such as the Mediterranean basin, the spectrum of thalassemia conditions is a continuum from asymptomatic (thalassemia minor), to patients with moderate but transfusion-independent anemia (thalassemia intermedia), to classical transfusion-dependent thalassemia major [1,2]. Some of the β-thalassemia phenotypic variations can be accounted for by interaction with different numbers of α-globin genes. The identification of factors

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TABLE I. Hematological and Molecular Data From Patients With Triplicated α-Globin Genes and Heterozygous β-Thalassemia

Patients	Age	Sex	β-Genotype	α-Genotype	β-Haplotype	−158 $^{G}\gamma$	Hb (g/dl)	MCV (fl)	MCH (pg)	Hb A_2 (%)	Hb F (%)	Blood transfusions	Splenomegaly (cm)
1[a]	26	M	IVS1-110/β^A	αα/ααα$^{3.7}$	I/II	C/C	9.8	72	21	5.9	6.4	No	3
2[a]	29	F	IVS1-110/β^A	αα/ααα$^{3.7}$	I/I	C/C	8.0	78	23	4.7	5.7	No	2
3[a]	21	F	β^{39}/β^A	αα/ααα$^{3.7}$	I/I	C/C	9.0	79	24	5.8	5.8	+	Splenectomy
4[a]	58	M	β^{39}/β^A	αα/ααα$^{3.7}$	ND	ND	8.1	77	25	4.4	2.1	++	8
5[a]	68	F	β^{39}/β^A	αα/ααα$^{3.7}$	I/II	C/C	8.4	81	25	4.7	3.0	++	Splenectomy
6	56	M	β^{39}/β^A	αα/ααα$^{3.7}$	I/II	C/C	6.5	78	24	5.8	1.5	+++	10
7	46	F	β^{39}/β^A	αα/ααα$^{3.7}$	ND	ND	9.5	60	19	4.3	4.7	No	4
8	37	F	β^{39}/β^A	αα/ααα$^{3.7}$	ND	ND	8.4	61	19	4.8	4.2	No	2
9	9	M	IVS1-1/β^A	αα/ααα$^{3.7}$	ND	C/C	8.0	60	19	4.7	4.9	No	Splenectomy
10[b]	26	M	β^{39}/β^A	αα/ααα$^{3.7}$	II/IX	C/T	11	65	25	2.7	13.6	No	5
11[b]	24	F	β^{39}/β^A	αα/ααα$^{3.7}$	II/IX	C/T	10	67	23	2.5	10.5	No	6
12	36	F	IVS1-1/β^A	αα/ααα$^{3.7}$	ND	C/C	10	60	20.4	5.1	1.6	No	Normal
13	22	F	IVS1-5/β^A	αα/ααα$^{3.7}$	ND	C/T	10.5	59	18	4.5	5.0	No	Normal
14	20	M	IVS1-5/β^A	αα/ααα$^{3.7}$	ND	C/C	11.2	66	20.5	4.5	4.9	No	Normal
15[a]	29	F	β^{39}/β^A	αα/ααα$^{3.7}$	V/IX	C/T	11.8	63	20	4.7	4.9	No	Normal
16[a]	35	M	IVS1-110/β^A	αα/ααα$^{3.7}$	I/I	C/C	12.7	62	19	5.1	<0.9	No	Normal
17	21	F	δβ/β^A	αα/ααα$^{3.7}$	ND	C/T	11.9	73	23.9	2.5	25	No	Normal

[a]Patients reported previously [9].
[b]δ^{+27} thalassemia trait.
ND, not determined; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin.

that modulate the expression of the disease is relevant to our understanding of pathophysiology and to genetic counseling.

High-sequence homology within the α-globin gene cluster often leads to misalignment and unequal homologous recombination events, producing a chromosome containing a triplicated α-globin gene (ααα) [3–5]. Two different α-gene triplications have been described: ααα$^{anti\text{-}3.7}$, which is most prevalent in Mediterraneans and Africans [6], and ααα$^{anti\text{-}4.2}$, which has been mainly described among Asians [7].

Homozygotes for β-thalassemia who inherit an α-thalassemia determinant have a less severe clinical course, while β-thalassemia heterozygotes who inherit a triplicated α-globin gene may have a more severe clinical course than thalassemia minor because of increased chain imbalance [2]. In a previous report on a large number of Italian thalassemia intermedia patients, we identified a small number of subjects heterozygous for β-thalassemia who presented with a more severe phenotype [8]. Some of these cases were found to be carriers of a triplicated α-globin gene. This report analyzes the clinical phenotypes in a large cohort of patients with coexistence of triplicated α-globin genes and a mutation in the β-like globin gene cluster. Seventeen patients with α-globin gene triplication have been identified, 16 of whom are heterozygous for β-thalassemia, and one who has a still undefined form of δβ-thalassemia. The β-globin gene defect in these patients is characterized and their clinical phenotype described in an effort to define how a triplicated α-globin gene can modify expression of β-thalassemia.

MATERIALS AND METHODS

Subjects

Seventeen patients were referred to the Children's Hospital of Philadelphia, or to the Clinical Division of the Dipartimento di Scienze Biomediche ed Oncologia Umana, Università di Torino, Torino, Italy, for evaluation of moderate to severe anemia with splenomegaly (11 patients), mild anemia and/or increased Hb A_2 and Hb F (6 patients). Among the patients, there were two pairs of siblings (patients 10 and 11 and patients 13 and 14) and one group of each father/daughter (patients 3 and 4) and mother/son (patients 9 and 12). Family studies included parents and all available relatives (total of 9 kindreds). Seven patients have been previously reported [9] (Table I), but their β-globin genotype was not previously characterized.

Hematological and Molecular Studies

Routine hematology and Hb analyses were performed following standard procedures. Determination of Hb included DEAE-cellulose microchromatography for quantitation of Hb A_2 and alkali denaturation for quantitation of Hb F. Ratios of non-α/α-globin chain synthesis were determined when possible by reverse-phase high-performance liquid chromatography (RP-HPLC). Genomic DNA was isolated from peripheral blood leukocytes [10], and determination of α-globin gene number was assessed as described previously [6]. Presence of the ααα$^{anti\text{-}4.2}$ allele was excluded by Southern gene blot analysis. The ααα$^{anti\text{-}3.7}$ allele was confirmed by polymerase chain reaction (PCR) amplification as previously described [11]. Two nondeletional forms of α-thalasse-

mia were screened for using *Nco*I digestion of PCR products from the α_2- and α_1-globin genes [12,13]. In addition, presence of the pentanucleotide deletion at the IVS-I donor site of the α_2-globin gene was also tested [14]. Common β-globin gene mutations in the Mediterranean area (β^{39}: C → T, IVS-I nt110: C → A, IVS-I nt1: G → A) were identified by allele-specific oligonucleotide analysis (ASO) [15], while rarer defects were screened by denaturing-gradient gel electrophoresis (DGGE) and then characterized by direct DNA sequence analysis of PCR products from genomic DNA [16]. β-Globin gene haplotypes were defined using a previously described PCR-based technique [17,18]. Presence of thymidine at position −158 in the 5′-flanking region of the $^{G}\gamma$ gene was defined after *Xmn*I digestion of a PCR product encompassing this region [18]. Screening for other mutations in the γ-globin gene promoters was performed by DGGE when indicated [19]. Deletions within the β-like globin gene cluster, including the Corfu deletional form of δ°-thalassemia were screened for using previously described PCR-based strategies [20,21]. Mutations in the δ-globin gene were assessed first by PCR amplification and restriction enzyme digestion as previously described [22–24] and then by automated DNA sequence analysis.

RESULTS

Genotypes and hematological profiles of the patients are summarized in Table I. All had the $\alpha\alpha\alpha^{anti\text{-}3.7}$ allele; 16 patients were also heterozygotes for a β-thalassemia mutation.

Eleven of 16 patients with a β-globin gene mutation (patients 1–11) have anemia of variable severity, as well as moderate to marked splenomegaly. Three patients (patients 3, 5, and 9) underwent splenectomy with improvement of their clinical status. Hb values were with the range of 6.5–11.0 g/dl, with a median of 8.7 g/dl. Usually at older ages, four of 11 patients developed requirements for either occasional (two individuals) or regular (two individuals) red blood cell transfusions. β-Globin gene mutations in this group were either the β° mutation at codon 39 (eight individuals) or the severe β^+ mutation at IVS-I-nt110 (two individuals), which are the most common mutations in the Mediterranean area. One patient (patient 9) of Northern European descent is heterozygous for a β°-thalassemia mutation in IVS-I-nt1. Family studies were available in five cases of this group (1, 2, 5, 6, 10, and 11). Analysis of the hematological phenotype of β-thalassemia carriers who did not inherit the triplicated α-gene (a total of nine cases) indicates that the extra α-gene, when inherited in association with β-thalassemia trait, is responsible for increased severity of the phenotype (data not shown).

Six patients are thalassemia minor carriers (patients 12–16) and present with mild anemia (Hb 10–12.7 g/dl), low red blood cell indices, and normal spleen size. Three of them (patients 13, 14, and 15) had Hb F levels higher than expected for β-thalassemia trait (approximately 5%). Two asymptomatic siblings (patients 13 and 14) are heterozygous for the IVS-I-nt5 G → C mutation. IVS-I-nt110, IVS-I-nt1, or β^{39} mutations were found in one patient each. Coexisting nondeletional forms of α-thalassemia in these patients would explain the milder phenotype compared to the other patients with the same β-globin gene genotype. Mutation in the translation initiation codon for α_2- [12] or α_1-globin mRNA [12] and a pentanucleotide deletion in the IVS-I donor site of the α_2-globin gene [13] were not observed in any of these cases. Patient 17 is a white female of Northern European origin with mild hypochromic anemia without marrow expansion or organomegaly. A δβ-thalassemia mutation is suspected based on the observed elevated Hb F levels with heterogeneous distribution and hypochromic, microcytic RBC indices. The exact β-like globin gene defect has not been characterized in this individual, who tested negative for HPFH-1, HPFH-2, HPFH-3, Spanish (δβ)° thalassemia, Hb Lepore, Sicilian (δβ)° thalassemia, Chinese $^{G}\gamma(^{A}\gamma\delta\beta)°$ thalassemia, Asian-Indian inversion-deletion $^{G}\gamma(^{A}\gamma\delta\beta)°$ thalassemia and the Turkish inversion-deletional form of (δβ)° thalassemia.

Four heterozygotes for the triplicated α-globin gene and normal β-globin genes were identified, all of whom had a normal hematological phenotype. Two families of particular interest are discussed below, one of which emphasizes variable effects of the α-globin gene triplication in the same family in individuals with identical β-globin genotypes.

In the first family, studies showing inheritance of the triplicated α-globin locus with β- and δ-thalassemia are shown in Figure 1. Two siblings (patients 10 and 11) are heterozygous for the β^{39} mutation and present with a more severe anemia and enlarged spleen, as compared with the father, who is also heterozygous for β-thalassemia. In addition, they show an unusual phenotype with normal Hb A_2 and elevated Hb F levels. A G → T substitution was found in patient 10 in the first nucleotide of codon 27 of the δ-globin gene, which results in an amino acid change (Ala → Ser), and which also presumably activates a cryptic splice site causing δ^+ thalassemia (δ^{+27}). Presence of the δ-globin gene mutation was confirmed by *Hae*III digestion of the amplified 5′ end of the δ-gene in patients I-2, 10, 11, and II-3 (Fig. 1). In addition, DNA sequence analysis of the two δ-genes from patient 10 showed homozygosity for the presence of a neutral polymorphic change at position 540 (T → A), a C deletion at 548 in IVS-II, and a TC → CT change at position 291–292 in IVS-II of both genes. The β^{39} mutation in this family resides on a β-like globin gene cluster haplotype II background, while the δ^{+27} mutation *in trans* resides on a haplotype IX background. The −158 $^{G}\gamma$ C → T

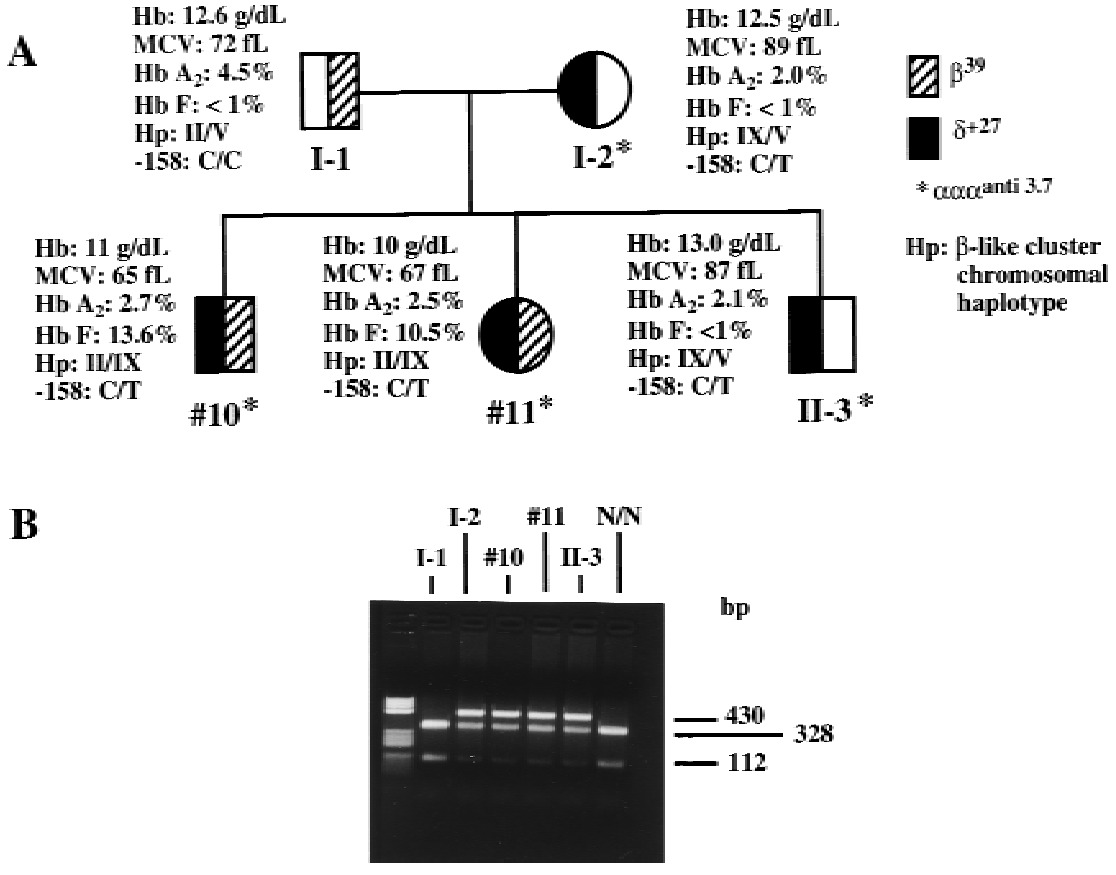

Fig. 1. Interaction of α-globin gene triplication with β^{39} nonsense and δ^{+27} mutations. A: Inheritance patterns show two generations with affected individuals indicated by solid or hatched half-circles or squares. Asterisk (*) indicates presence of triplicated α-globin gene. B: Agarose gel electrophoresis of *Hae*III-restricted PCR products showing presence of a 430-bp product in δ^{+27} individuals (patients I-2, 10, 11, and II-3). Normal individuals (I-1 and N/N) show the presence of two bands (328 and 112 bp).

change was detected in the mother and in all children (patients 10, 11, I-2, and II-3) and is found on a haplotype IX background. A 4-bp deletion (AGCA) in the $^{A}\gamma$-gene promoter region, which resides on a haplotype II background, was found in the father (I-1) and in patients 10 and 11. Coexistence of α-triplication and δ^{+27} thalassemia results in normal Hb A_2 and Hb F levels (patients I-2 and II-3) (Fig. 1).

In the second family (patients 9 and 12), several unanticipated findings are not readily explained by genotype/phenotype analysis. For example, although patient 9 and his mother (patient 12) have the same α- and β-globin genotypes ($\alpha\alpha/\alpha\alpha\alpha^{anti\text{-}3.7}$, $\beta^{IVSI\text{-}1}/\beta^{A}$) and a similar non-α/α-chain ratio (0.37 and 0.35, respectively), patient 9 has a more severe phenotype compared to his mother. Deletional and nondeletional forms of α-thalassemia (e.g., initiation codon mutations or a pentanucleotide deletion at the IVS-I splice donor site) were excluded in patient 12 and the father (data not shown). We therefore have no explanation for the discrepancy in phenotypes comparing patients 9 and 12, who have similar globin genotypes.

DISCUSSION

Earlier studies showed the additional α-globin gene in individuals with a triplication results in expression of excess α mRNA and α-globin chains [3,5]. Studies of the duplicated and triplicated α-globin genes show the α_2-globin gene predominates in expression over α_1 with observed normal α_2/α_1 mRNA ratios of ~3:1 [25,26]. It is believed that if α-globin chain excess were small, proteolysis would occur early in erythroid development, protecting the erythroid precursors from an early death. Thus, it is not surprising that patients heterozygous for triplicated α-globin genes are hematologically normal [27]. Precipitation would occur, however, in cases of high α-globin chain excess, as in individuals who also inherit a defective β-globin gene.

From the few isolated cases reported so far, clinical phenotypes of compound heterozygotes with excess α-globin genes and a β-thalassemia mutation usually correlate with the number of excess α-genes. Combination of a β-thalassemia mutation with two extra α-globin genes either *in trans* (ααα/ααα) or *in cis* (αα/αααα)

usually causes thalassemia intermedia [27–31]. In this way, the extra α-gene behaves as a modifier gene [32]. Modifier genes can be defined as mutant genes that lack any phenotypic effect in normals but that are able to modify the biochemical or clinical expression of a second mutant gene [32]. The extra α-gene is indeed silent in the simple heterozygotes for the triplication but becomes manifest in association with heterozygous β-thalassemia. Interaction with a single extra gene can result in a more severe phenotype, especially if the β-globin gene defect is a β°- or severe β^+-thalassemic mutation, such as IVS-I-nt110 [9,32–35]. However, a triplicated α-globin gene may alter clinical severity of β-thalassemia heterogotes in such a mild way that the influence is not detectable [9,27,31,36].

This study presents the largest series of patients with these complex genotypes. Their clinical phenotypes range from thalassemia minor to thalassemia intermedia of moderate severity. Triplicated α-genes inherited with β°-thalassemia or severe β^+-thalassemic mutations were predominantly, but not exclusively, associated with increased severity. Comparison of the phenotype with β-thalassemia carriers within the same families in five cases led to the conclusion that inheritance of the triplicated α-gene increased clinical severity. However, we found patients with severe β-thalassemia mutations interacting with a triplicated α-gene whose hematological phenotype was that of a β-thalassemia carrier. Moreover, differences in phenotypic expression in family members with the same α-gene triplication/β-thalassemia mutation (patients 9 and 12) remains unexplained. It is likely that the delicate balance between α- and β-chains is influenced by other factors (e.g., different degrees of proteolysis) that are currently not amenable to molecular analysis. Genes coding for these factors may behave as sequence modifiers that at present are not identifiable. It is unclear what proportion of β-thalassemia carriers with the triplication have a mild phenotype, since extensive studies are lacking. In our series, the results may be biased by selection of patients with more severe phenotypes.

Two of our patients (patients 10 and 11) with the triplication were also heterozygous for the β°-codon 39 mutation associated with haplotype II (Fig. 1) and presented with Hb F levels significantly higher than those in other heterozygotes with the codon 39 mutation in our study. In addition, these two patients presented with normal Hb A_2 levels in spite of the presence of a β^{39} mutation. Both individuals were found to have a δ-globin mutation *in trans* to the β^{39} mutation. Although this finding explains the lack of elevated Hb A_2 levels in patients 10 and 11, the reason for elevated production of Hb F remains unclear. Association of β^{39} and δ^{+27} thalassemia in Sardinia results in a thalassemia trait with normal Hb A_2 but without increased Hb F levels [13]. β-Cluster haplotype-linked determinants could have resulted in elevated Hb F. The δ-thalassemia mutation in these patients occurs on a haplotype IX β-gene cluster chromosomal background with a thymidine at −158 bp 5′ of the $^{G}\gamma$ gene, which has been shown to be associated with increased expression of the γ-gene in individuals with decreased β-globin gene expression. *In trans* they have a 4-bp deletion in the $^{A}\gamma$-globin gene 5′-flanking region that was previously shown to be associated with decreased $^{A}\gamma$-globin chains [37]. It is likely that this deletion has no role in increasing Hb F expression in these patients. Co-inheritance of a triplicated α-globin gene increases the α-globin pool and, in the presence of a β-cluster haplotype that has been associated with increased Hb F production [38], may favor increased production of Hb F.

The study of this family is an example of the genetic complexity that can be observed in a monogenic disease [39]. In addition, results for subjects I-2 and II-3 show that hematologically normal subjects may have ''silent'' mutations in the α- and β-globin gene clusters that may manifest clinically only when co-inherited with a β-thalassemia allele. Additional genetic determinants with similar effects may exist that can modify phenotype in individuals sharing the same β-globin gene mutation.

Our results and those of others show that the triplicated α-globin gene may accentuate the imbalance of chain synthesis caused by a defective β-globin gene, although this is not always true. Thus, in regions endemic for β-globin gene mutations, the presence of the triplicated α-globin gene chromosome might have a selective disadvantage compared to the (−α) allele, which may, at least in part, explain differences in prevalence between triplicated and single α-globin gene-containing chromosomes. Interaction with triplicated α-genes should be considered in patients with heterozygous β-thalassemia who present with a more severe phenotype than expected. The potentially negative effects of the triplicated α-globin gene, when coinherited with β thalassemia, need to be considered in the clinical care of these individuals, as they may develop transfusion requirements or significant splenomegaly.

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